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Homotypic Interactions Mediated by Slamf1 and Slamf6 Receptors Control NKT Cell Lineage Development

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SUMMARY

Commitment to the T and natural killer T (NKT) cell lineages is determined during αβ T cell receptor (TCR)-mediated interactions of common precursors with ligand-expressing cells in the thymus. Whereas mainstream thymocyte precursors recognize major histocompatibility complex (MHC) ligands expressed by stromal cells, NKT cell precursors interact with CD1d ligands expressed by cortical thymocytes. Here, we demonstrated that such homotypic T-T interactions generated "second signals" mediated by the cooperative engagement of the homophilic receptors Slamf1 (SLAM) and Slamf6 (Ly108) and the downstream recruitment of the adaptor SLAM-associated protein (SAP) and the Src kinase Fyn, which are essential for the lineage expansion and differentiation of the NKT cell lineage. These receptor interactions were required during TCR engagement and therefore only occurred when selecting ligands were presented by thymocytes rather than epithelial cells, which do not express Slamf6 or Slamf1. Thus, the topography of NKT cell ligand recognition determines the availability of a cosignaling pathway that is essential for NKT cell lineage development.

INTRODUCTION

Natural killer T (NKT) cells constitute a separate lineage of innate-like T lymphocytes involved in various infectious, autoimmune, allergic, and cancerous conditions (Bendelac et al., 2006; Godfrey et al., 2004). They emerge from the thymus as memory or effector cells that explosively release T helper 1 (Th1) and Th2 cytokines and chemokines upon recognition, through conserved semi-invariant αβ T cell receptors (TCRs), of glycolipid ligands presented by the major histocompatibility complex (MHC)-like molecule CD1d. There is growing evidence indicating that differential signaling associated with TCR recognition of ligands at the $CD4^+CD8^+$ double-positive (DP) stage of thymic development underlies the divergence between NKT cells, mainstream CD4 and CD8 T cells, regulatory T cells, and CD8αα cells (Benlagha et al., 2005; Gapin et al., 2001; Jordan et al., 2001; Leishman et al., 2002; Yamagata et al., 2004). However, the specific nature of these differences and their molecular basis have remained elusive.

NKT cells exhibit autoreactivity to CD1d-expressing cortical thymocytes (Bendelac, 1995), and the dominant subset expressing Vα14-Jα18 and Vβ8, 7, or 2 TCRs (Park et al., 2001) recognizes endogenous ligands such as iGb3 as weak agonists (Schumann et al., 2006; Zhou et al., 2004). In contrast, conventional T cells recognize thymic peptides presented by MHC proteins as partial agonists (Hogquist et al., 1994). Importantly, the development of NKT cells is unique because it relies on ligand expression by cortical thymocytes (Coles and Raulet, 2000; Schumann et al., 2005; Wei et al., 2005). Indeed, CD1d expression by cortical thymocytes alone was both required and sufficient in vivo for positive selection, lineage expansion, and differentiation into the NKT cell lineage. This could impact their lineage differentiation because some of the signals emanating from such homotypic thymocyte-thymocyte interactions during TCR recognition of ligands probably differ from those associated with heterotypic thymocyte-stromal cell interactions. Interestingly, the ectopic expression of MHC class II by the thymocytes of mice expressing the class II transactivator (CIITA) transgene driven by a CD4 promoter resulted in the selection of $CD4^+$ T cells expressing a memory or effector phenotype similar to that of NKT cells (Choi et al., 2005; Li et al., 2005). Furthermore, mice lacking Tec kinases developed memory or effector $CD8^+$ T cells that also resembled NKT cells in their expression of NK lineage markers and in their dependence on MHC class I ligand expression by bone marrow rather than epithelial cells (Atherly et al., 2006; Broussard et al., 2006).

Together, these observations suggest that unidentified signals provided by bone-marrow-derived selecting cell types might contribute to the differentiation of memory or effector lineages (Locksley, 2002).

Indirect evidence suggests that members of the signaling lymphocytic-activation molecule (SLAM) family of receptors might be involved in this process. These proteins, which are all encoded in the SLAM locus, are mostly homotypic self-associating receptors expressed by cells of hemopoietic origin (Engel et al., 2003; Veillette, 2006). They have recently emerged as TCR-dependent or -independent regulators of adhesion and cellular activation during interactions between mature T, B, macrophage, and dendritic cells, and they control several aspects of innate and adaptive responses, as well as chronic diseases (Cannons et al., 2006; Crotty et al., 2003; Howie et al., 2005; Kumar et al., 2006; Wang et al., 2004). Despite intricate patterns of expression of several of the SLAM family receptors during hematopoiesis (Kiel et al., 2005) and thymopoiesis, their role in lymphocyte development has not been demonstrated. In T cells and thymocytes, SLAM-receptor-initiated signaling is mediated in part by SLAM-associated protein (SAP, also called SH2D1A), an adaptor that recruits Src kinase Fyn, which in turn phosphorylates SLAM receptors. The receptors then serve as a docking site for a set of signaling molecules (Engel et al., 2003; Veillette, 2006). Mice lacking Fyn or SAP exhibited severe NKT cell defects, as did humans with the X-linked lymphoproliferative (XLP) syndrome associated with SAP mutation (Chung et al., 2005; Eberl et al., 1999; Gadue et al., 1999; Nichols et al., 2005; Pasquier et al., 2005). On the basis of these observations, it could be envisioned that a costimulatory pathway involving SAP and Fyn is recruited by SLAM family receptors at some critical stage of NKT cell development (Borowski and Bendelac, 2005). However, in mice lacking the SLAM family receptors Slamf6 (Ly108, NTBA), Slamf1 (SLAM, CD150), or Slamf3 (Ly9, CD229), NKT cell development seemed unperturbed (Graham et al., 2006; Howie et al., 2005; Wang et al., 2004). Other reports have suggested that Fyn could be activated downstream of TCR signaling (Hermiston et al., 2002) and that SAP could function without binding Fyn (Cannons et al., 2006; Chan et al., 2003; Gu et al., 2006; Howie et al., 2002; Simarro et al., 2004), raising the possibility that Fyn and SAP mediate in part separate pathways contributing to NKT cell development that might be independent of SLAM-family receptors. Thus, the precise pathways recruiting SAP and Fyn during NKT cell development remain enigmatic. In addition, the developmental stages at which they are required have not been characterized. Contrasting with the hypothesis that SAP and Fyn costimulate TCR signaling, the apparent restoration of NKT cell development in *Fyn*$^{-/-}$ mice by the transgenic expression of the canonical Vα14-Jα18 TCRα chain has suggested that Fyn acted at early stages of development upstream of TCR expression and therefore prior to thymic positive selection (Gadue et al., 2004).

Here we used genetic and bone-marrow chimera approaches to provide evidence for a linear pathway of signaling that is initiated by homophilic self association of Slamf1 and Slamf6 expressed by cortical thymocytes and is propagated by SAP and Fyn. Importantly, we showed that this pathway was recruited during the TCR signaling events driving the positive selection of NKT cell precursors. Furthermore, we demonstrated that, although unessential for positive selection, Slamf1 and Slamf6 signaling critically controlled the characteristic expansion and differentiation of the NKT cell lineage that follows thymic selection.

RESULTS

NKT Cell Developmental Arrest in the Absence of SAP and Fyn

We first examined whether the NKT cell defects in Fyn or SAP mutants preceded or followed Vα14-Jα18 TCR expression (Gadue et al., 2004). We found similar frequencies of canonical Vα14-Jα18 rearrangements in sorted DP thymocytes from wild-type, SAP- or Fyn-deficient mice (Figure 1A), ruling out a role of SAP or Fyn in the generation of the canonical NKT TCR α chain. We then directly analyzed the NKT cell developmental defects in different Fyn- and SAP-deficient mice. In Vα14-Jα18 transgenic mice, Fyn ablation reduced by 85% the frequency of mature NKT thymocytes identified by CD1d-αGalCer tetramers and low expression of the heat-stable antigen CD24 (Figure 1B). Furthermore, the ablation of SAP, which in SLAM-receptor signaling functions upstream of Fyn, induced a nearly complete block in the development of mature NKT cells. The less severe phenotype of Fyn mutants might suggest compensation by other Src kinases, such as Lck (Simarro et al., 2004). To extend these observations to nontransgenic thymocytes and further characterize the arrested stage, we stained rare developmental intermediates with allophycocyanin (APC)-conjugated CD1d-αGalCer tetramers and enriched them with anti-APC paramagnetic beads. This enrichment procedure allows the unambiguous detection by the fluorescence-activated cell sorting (FACS) of the few hundred CD24^{hi} Tetramer^{hi} CD69^{hi} NKT cell lineage precursors that are present in the normal thymus and represent the stage that immediately follows positive selection (Benlagha et al., 2005). As shown in Figure 1C, NKT cell development was arrested at the CD24^{hi} Tetramer^{hi} CD69^{hi} stage in both SAP- and Fyn-deficient thymocytes. The developmental block in Fyn-deficient mice again appeared to be leaky because rare cells (<1% of wild-type) reached the mature CD24^{lo} stage. The arrested cells were reduced in numbers compared with wild-type cells, and they expressed CD69, a marker of positive selection, albeit at a 66% reduced frequency. A similar arrest at the CD24^{hi} Tetramer^{hi} CD69^{hi} stage after positive selection by CD1d ligand was observed in Vα14-Jα18 transgenic thymocytes lacking SAP (Figure 1D). Together, these results demonstrate that the NKT cell developmental arrest in SAP- or Fyn-deficient mice occurs not only after TCR expression but also during or just after positive selection.

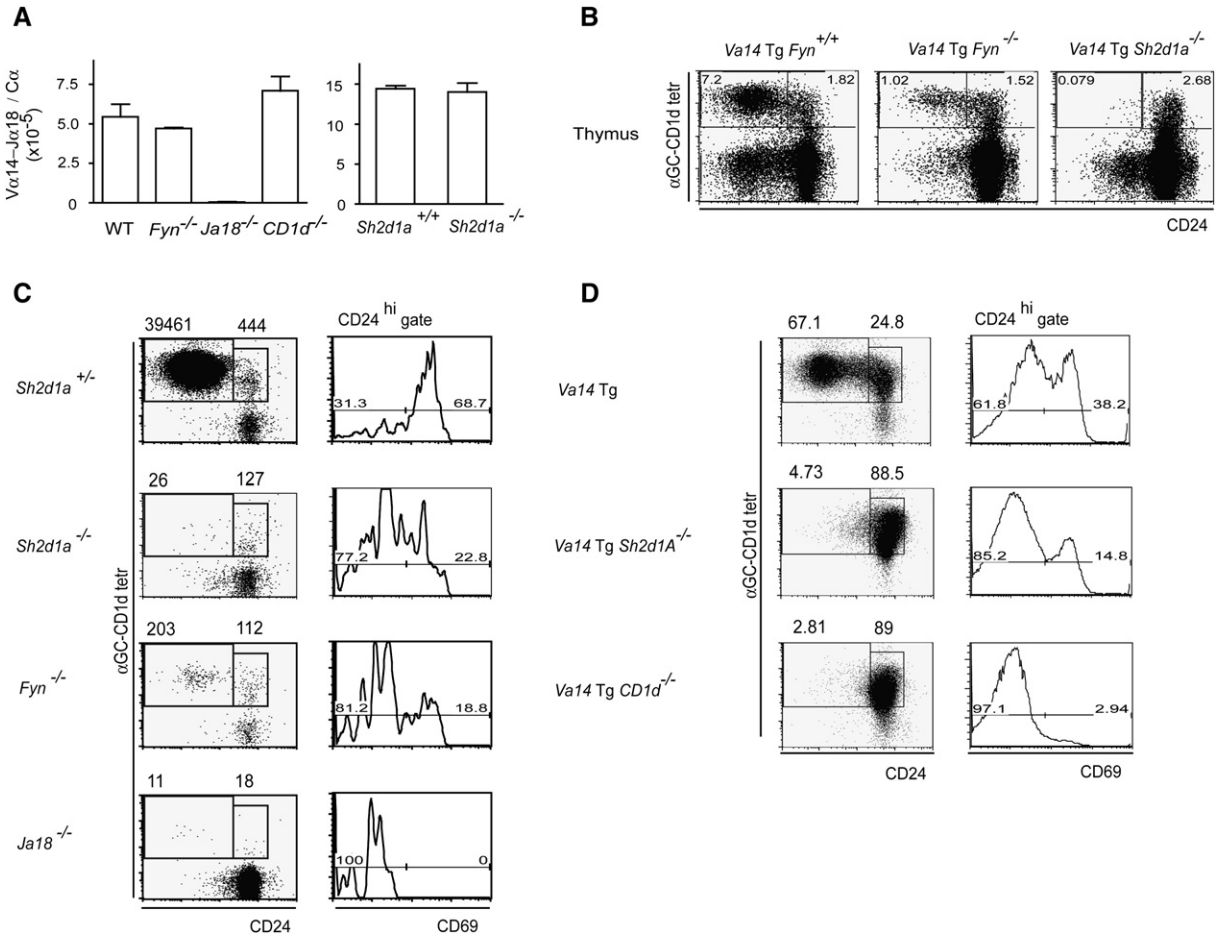

Figure 1. NKT Cell Developmental Arrest in SAP- and Fyn-Deficient Mice

(A) Conserved frequency of Vα14-Jα18 rearrangements in Fyn- and SAP-deficient thymocytes. Quantitative reverse transcriptase (RT)-PCR of canonical Vα14-Jα18 rearrangements and Cα in sorted DP thymocytes from WT, *Fyn*$^{-/-}$, *Jα18*$^{-/-}$, and *CD1d*$^{-/-}$ (left) and in DP thymocytes from *CD1d*$^{-/-}$ *Sh2d1a*$^{+/+}$ and *CD1d*$^{-/-}$*Sh2d1a*$^{-/-}$ mice (right). *Sh2d1a*$^{-/-}$ mice were crossed onto a *CD1d*$^{-/-}$ background so that any contamination of DP thymocyte by mature NKT cells could be eliminated. The values shown are ratios between Vα14-Jα18 and Cα transcripts (mean + standard deviation [SD], n = 3).

(B) *Vα14-Jα18* transgenic thymocytes on a *Fyn*$^{+/+}$, *Fyn*$^{-/-}$, or *SAP*$^{-/-}$ background were stained with CD1d-αGalCer tetramers and CD24 so that the immature CD24^{hi} and mature CD24^{lo} stages could be enumerated. Cell frequencies are indicated in the corresponding gates.

(C) CD1d-αGalCer tetramer-positive thymocytes were enriched by autoMACS from pools of thymi obtained from 2-week-old *Sh2d1a*$^{+/-}$ and *Sh2d1a*$^{-/-}$ littermates and *Fyn*$^{-/-}$ and *Jα18*$^{-/-}$ mice prior to FACS analysis with CD24 (left column). Numbers in the CD1d-αGalCer^{+} CD24^{hi} and CD24^{lo} gates represent absolute cell numbers recovered from two pooled thymi. Gated CD1d-αGalCer^{+} CD24^{hi} cells were analyzed for CD69 expression (right column). Percentages are indicated over corresponding brackets. Similar results were obtained in three independent experiments.

(D) Stage of NKT cell developmental arrest in the *Vα14* Tg *Sh2d1a*$^{-/-}$ thymus. CD1d-αGalCer tetramer^{+} thymocytes were enriched with paramagnetic beads and submitted to FACS analysis as in (C). One of two experiments with similar results is shown.

Thymic Expression Pattern of SLAM Family Receptors

We next addressed the question of whether and which SLAM family members might be involved in NKT cell development. The SLAM locus comprises genes encoding several SAP-binding family members that are conserved in mouse and human, including Slamf1 (SLAM, CD150), Slamf3 (Ly9, CD229), Slamf4 (2B4, CD244), Slamf5 (CD84), and Slamf6 (Ly108, NTBA) (Engel et al., 2003; Veillette, 2006). By using monoclonal antibodies, we found that Slamf4 was not expressed on mouse thymocytes (data not shown). Of the remaining receptors, Slamf1 and Slamf6 displayed the highest expression on DP cortical thymocytes with conserved or decreased expression on mature single-positive (SP) thymocytes, a pattern similar to CD1d (Roark et al., 1998), whereas Slamf3 and Slamf5 had lower expression on DP than SP cells (Figure 2A). Developing NKT thymocytes had a similar pattern of expression, with Slamf1 being rapidly downregulated after the DP stage, whereas Slamf6 expression persisted longer, until the CD24^{lo} stage (Figure 2B). Furthermore, Slamf1 and Slamf6 were conspicuously absent

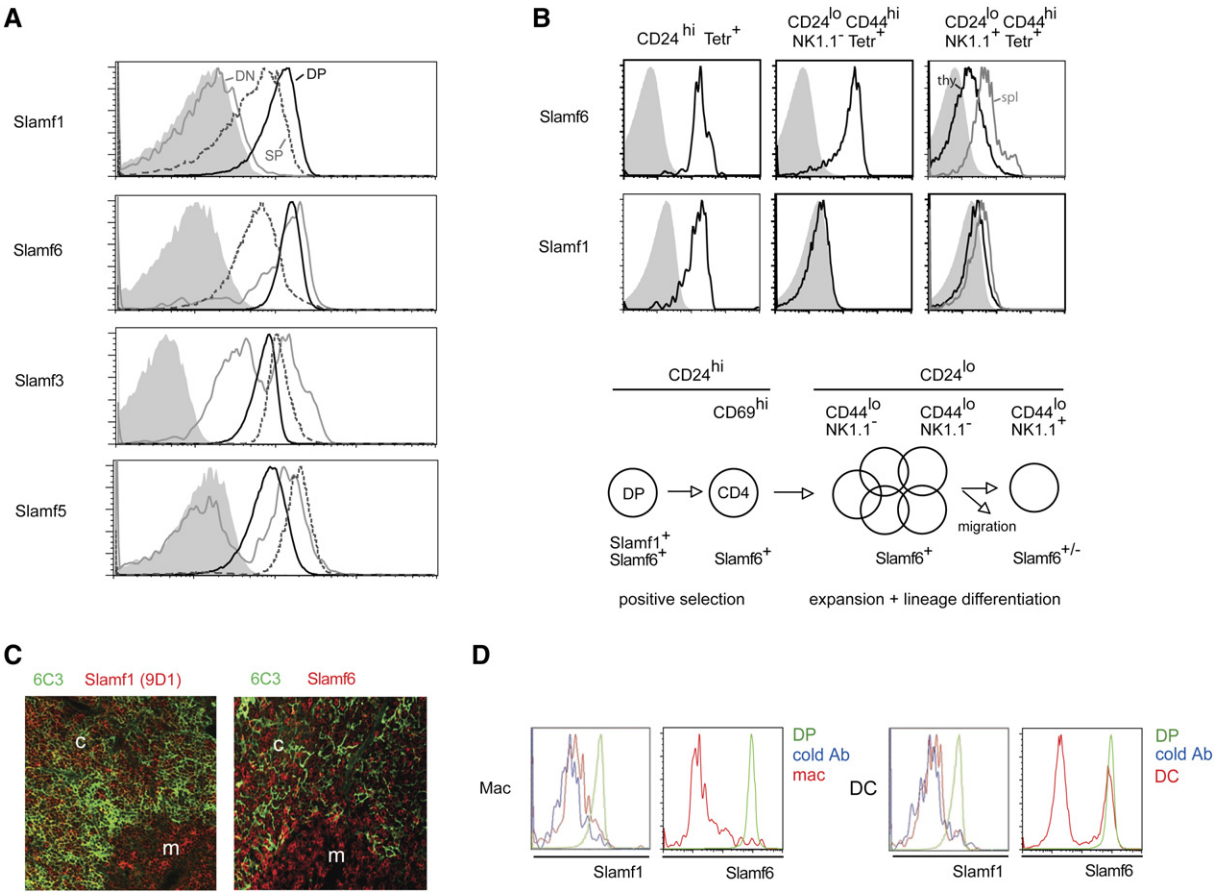

Figure 2. Expression Pattern of SLAM Family Members on Thymocytes and NKT Cells

(A) Double negative (DN), double positive (DP) and mature single positive (SP) thymocytes from B6 mice (or BALB/c for Slamf3 [Ly9]) were analyzed for the expression of SLAM family receptors. DP cells are represented by a solid black line, SP cells by a dashed dark gray line, and DN cells by a light gray line, as indicated. Shaded profiles represent isotype controls or staining of thymocytes lacking the corresponding gene or epitope.

(B) Slamf1 and Slamf6 expression at different stages of NKT thymocyte development. Left panels show immature $CD24^{hi}$, middle panels show mature $CD24^{lo}CD44^{hi}NK1.1^{-}$, and right panels show terminally differentiated $CD24^{lo}CD44^{hi}NK1.1^{+}$ cells in the thymus (black) and spleen (gray). The findings are summarized in the NKT cell developmental chart below the FACS panels.

(C) Immunohistochemical staining of thymi for Slamf1 (left) and Slamf6 (right) expression (in red) as well as cortical epithelial cells (antibody 6C3 in green).

(D) Flow cytometry analysis of thymic $CD11b^{+}$ macrophages and $CD11c^{+}$ dendritic cells (red) compared with DP thymocytes (green). The blue profile corresponds to a staining with biotin-conjugated antibody after incubation with excess unconjugated (cold) antibody (negative control). One of three experiments with similar results is shown.

from other thymic cell types, including epithelial cells, dendritic cells, and macrophages, with the exception of a subset of $CD11c^{+}$ cells expressing Slamf6 (Figures 2C–2D).

NKT Cells in Slamf1- or Slamf6-Deficient Mice

By using flow cytometry, we compared the thymus, spleen, and liver lymphocytes of *Slamf6*$^{+/-}$ and *Slamf6*$^{-/-}$ littermate pairs as well as *Slamf1*$^{+/-}$ and *Slamf1*$^{-/-}$ littermate pairs and found little difference in NKT cell numbers in most cases. These results are consistent with previous reports of *Slamf6*- (Howie et al., 2005), *Slamf1*- (Wang et al., 2004) or *Slamf3*- (Graham et al., 2006) deficient mice. The compilation of data did suggest a modest reduction (~50%) in the case of *Slamf6*$^{-/-}$ mice (Figure 3).

To reveal latent, intrinsic NKT cell defects associated with these mutations, we generated radiation chimeras in which lethally irradiated Jα18-deficient hosts were competitively reconstituted with a 1:1 mixture of wild-type and Slamf1- or Slamf6-deficient bone marrow cells expressing different marker alleles of CD45 for convenient FACS identification. In all these chimeras, the knockout (KO):WT ratio of thymocytes and splenocytes ranged between 0.3 and 0.7, demonstrating the similar capacity of WT and KO bone marrow cells for general lymphocyte reconstitution. Significant NKT cell decreases of ~50%–75% on average were observed for both the Slamf1- and the Slamf6-deficient populations compared with the wild-type (Figure 4A). Because the Slamf1-deficient and the Slamf6-deficient bone marrows used in these

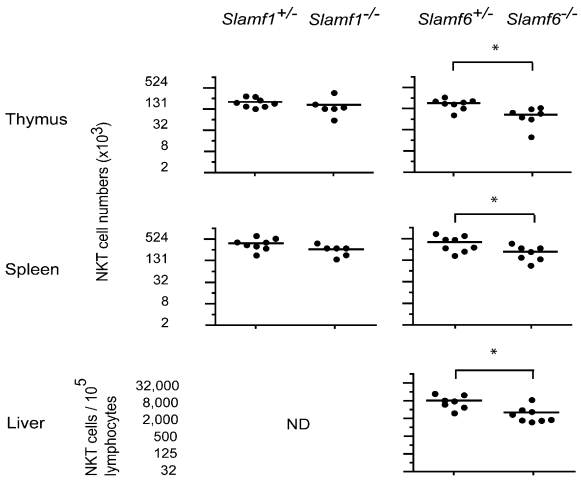

Figure 3. Individual Contributions of Slamf1 and Slamf6 to NKT Cell Development

Scatter plots showing absolute numbers (log_2 scale) of CD1d-αGalCer-positive cells in individual thymi, spleens, and livers of 3–5-week-old *Slamf1*$^{+/-}$ and *Slamf1*$^{-/-}$ littermates and *Slamf6*$^{+/-}$ and *Slamf6*$^{-/-}$ littermates. Because of variations in liver lymphocyte recovery between experiments, liver NKT cell numbers are enumerated per 10^5 lymphocytes. Liver NKT cells were not examined in *Slamf1*$^{-/-}$ mice ("ND" indicates not determined). "*" indicates statistical significance ($p < 0.05$) with an unpaired t test.

chimeras were from mice that had been originally derived from embryonic stem cells of 129 origin and were subsequently backcrossed to B6, the possibility existed that these relatively modest differences resulted from some heterogeneity between the SLAM locus of 129 and B6. We thus generated control mixed chimeras with mutations of *Slamf4* and *Slamf2*, which encode 2B4 (CD244) and its ligand CD48, respectively. Both proteins are encoded in the same SLAM locus, but they should not affect NKT cell development because, whereas Slamf2 is highly expressed by all immature and mature thymocytes, Slamf4 is not expressed in the thymus or during NKT cell development (data not shown). The Slamf4 mutants were derived from embryonic stem cells of B6 origin, and the Slamf2 mutants were derived from embryonic stem cells of 129 origin and backcrossed onto the B6 genetic background. In such control (*Slamf4*$^{-/-}$ + *Slamf6*$^{-/-}$) or (*Slamf2*$^{-/-}$ + *Slamf6*$^{-/-}$) mixed chimeras, the development of NKT cells lacking Slamf6 was as defective relative to NKT cells lacking Slamf4 or Slamf2 as it was relative to wild-type NKT cells in the (WT + *Slamf6*$^{-/-}$) mixed chimeras (Figure 4A). An additional set of control (WT + *Slamf2*$^{-/-}$) chimeras directly demonstrated that the SLAM locus from 129 did not contribute to the NKT cell defects observed in *Slamf1*$^{-/-}$ or *Slamf6*$^{-/-}$ mice (Figure 4A).

A more detailed analysis of the NKT cell developmental stages in these mixed chimeras revealed a clear defect at the transition between the CD24^{hi} and CD24^{lo} stages in the Slamf1- or Slamf6-deficient cells compared with the wild-type (Figure 4B). This transition corresponds to the phase of expansion of the NKT cell lineage after positive selection has occurred. Thus, both Slamf1 and Slamf6 contribute to the development of NKT cells after positive selection.

Slamf1 and Slamf6 Double Deficiency

Owing to their overlapping expression pattern and their shared usage of SAP and Fyn for intracellular signaling, Slamf1, Slamf6 and some of the other SLAM family receptors might be partly redundant in the thymus, as well as in peripheral immune responses. Because they are encoded in the same locus, however, double- or triple-mutant mice could not be generated by the breeding of single mutants. Instead, we reconstituted lethally irradiated Jα18-deficient hosts with a 1:1 mixture of CD45-allele-marked CD1d-deficient bone marrow that lacked Slamf6 (*Slamf6*$^{-/-}$*CD1d*$^{-/-}$) and CD1d-sufficient bone marrow that lacked Slamf1. In these chimeras, NKT cell precursors originating from the *CD1d*$^{-/-}$ compartment lacked Slamf6 and were forced to engage their TCR on CD1d-expressing thymocytes that lacked Slamf1, creating a special "double-mutant" situation during these cell encounters because neither Slamf1 nor Slamf6 could engage in homophilic self-interactions (Figure 5A, top). These *Slamf6*$^{-/-}$*CD1d*$^{-/-}$ precursors, however, were able to receive Slamf1 signals from bystander thymocytes in *trans*. In contrast, the *Slamf1*$^{-/-}$ cells had to interact with other *Slamf1*$^{-/-}$ cells for their TCR to see CD1d, creating a single-mutant situation (Figure 5A, bottom). In the thymus and peripheral tissues of these chimeras, the double-mutant cells generated on average six to ten times less NKT cells than did the single-mutant cells (Figures 5B and 5C, first column). Control chimeras expressing a 1:1 mixture of wild-type and *CD1d*$^{-/-}$ bone marrow demonstrated that the absence of CD1d on half of the thymocytes did not interfere with NKT cell development (Figures 5B and 5C, fourth column). Notably, in these mixed chimeras, as in those shown in Figure 4A comparing *Slamf2*$^{-/-}$ and *Slamf6*$^{-/-}$, the NKT cell precursors shared the same SLAM locus of 129 origin and differed only with respect to the *Slamf1* and *Slamf6* mutations. Taking into account the fact that the *Slamf1*$^{-/-}$ cells, against which the *Slamf6*$^{-/-}$*CD1d*$^{-/-}$ cells were competing, were themselves impaired by 50% when compared with wild-type (Figure 4), the findings suggest that the combined interruption of homophilic Slamf1-Slamf1 and Slamf6-Slamf6 interactions between the CD1d-presenting thymocyte and the NKT precursor results in a 92%–95% reduction in NKT cell development that cannot be rescued by "bystander" interactions with other thymocytes expressing these receptors. As expected from previous studies of SAP- and Fyn-deficient mice, the development of conventional CD4$^+$ and CD8$^+$ T cells was not altered in the mixed chimeras (data not shown).

A second set of mixed chimeras was constructed with a 1:1 mixture of *Slamf1*$^{-/-}$ and *Slamf6*$^{-/-}$ bone marrow cells. In these chimeras, a NKT cell precursor lacking Slamf1, for example, will be deprived of Slamf1 interactions

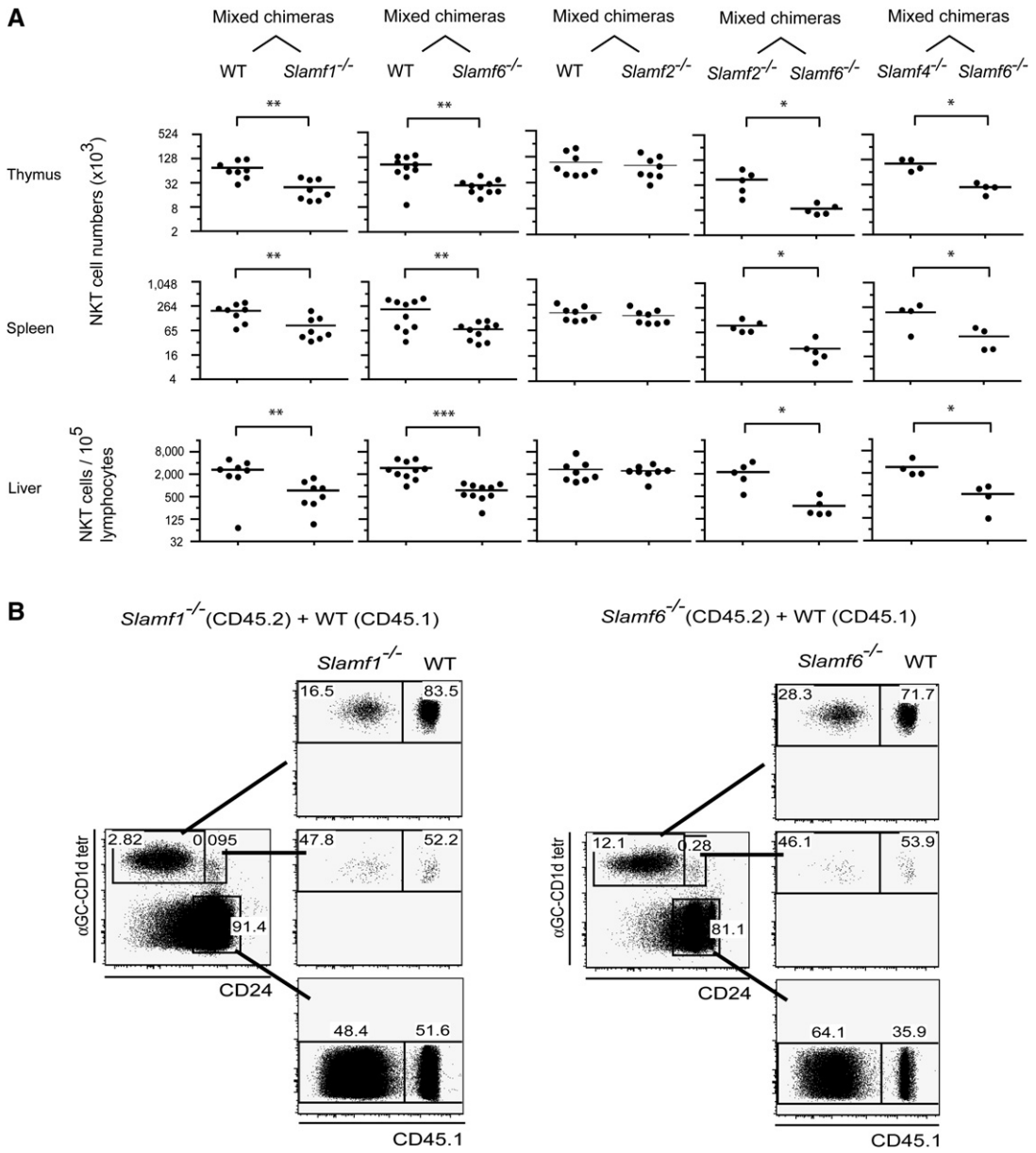

Figure 4. NKT Cell Development in Competition Chimeras

(A) 1:1 mixtures of WT + *Slamf1*$^{-/-}$, WT + *Slamf6*$^{-/-}$, WT + *Slamf2*$^{-/-}$, *Slamf4*$^{-/-}$ + *Slamf6*$^{-/-}$, and *Slamf2*$^{-/-}$ + *Slamf6*$^{-/-}$ bone marrow were injected into lethally irradiated *Jα18*$^{-/-}$ hosts, and chimeras were analyzed at 6–8 weeks. Absolute NKT cell numbers were calculated for each CD45 allele-marked compartment and were then divided by the number of lymphocytes in the corresponding CD45 allele-marked fraction and multiplied by the total number of lymphocytes to adjust for differences between the ratio of reconstitution by the two bone marrows which ranged between 0.3 and 0.7. "*," "**," and "***" indicate statistical significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively) with a t test for paired comparisons (2 experiments are pooled as they showed similar results [ANOVA]).

(B) FACS analysis of MACS-enriched CD1d-αGalCer tetramer^{+} CD24^{hi} and CD24^{lo} cells originating from the WT and mutant (CD45 allele-marked) bone marrows. The chimeric distribution of the tetramer-negative thymocytes is also shown (bottom dot plots).

permanently but will also miss Slamf6 interactions in 50% of its cellular interactions because the other half of the thymocytes lacked Slamf6. Interestingly, these competitive chimeras suggested that the impact of Slamf6 might be more important than that of Slamf1 because on average the absence of Slamf6 resulted in 50%–75% less NKT cells than did the absence of Slamf1 (Figures 5B and 5C, second column). This might be due to the persisting expression of Slamf6 after the DP stage (Figure 2B) because it is known that NKT cell precursors continue to engage their TCR for an extended period of time until terminal maturation into NK1.1^{+} cells (McNab et al., 2005). In addition,

statistical comparison between the two sets of mixed chimeras in the first and second columns of Figures 5B and 5C showed that the lack of CD1d in the *Slamf6*$^{-/-}$ compartment further increased the magnitude of the NKT cell defects in comparison with the *Slamf1*$^{-/-}$ compartment in different tissues by ~3-fold on average (Figure 6) ($p < 0.001$, analysis of variance [ANOVA]), demonstrating that functionally relevant self-interactions between SLAM family members occurred at the same time as TCR engagement and with the same CD1d-expressing thymocyte. This conclusion was further supported by reverse chimeras made of a 1:1 mixture of *Slamf6*$^{-/-}$ and *Slamf1*$^{-/-}$*CD1d*$^{-/-}$ bone marrows (Figures 5B and 5C, third column) in which the functionally double-mutant *Slamf1*$^{-/-}$*CD1d*$^{-/-}$ population now generated less NKT cells than did the single-mutant *Slamf6*$^{-/-}$ population. The statistical comparison of the two sets of mixed chimeras in the second and third columns of Figures 5B and 5C showed that the ablation of CD1d in the *Slamf1*$^{-/-}$ cell population, functionally changing these cells into double mutants, increased the magnitude of its NKT cell defect by 6-fold on average in different tissues (Figure 6) ($p < 0.001$, ANOVA). The ratios of NKT cells developing in the different compartments of individual mixed chimeras are summarized in Figure 6. Altogether, these results reveal the partial redundancy of Slamf1 and Slamf6 and demonstrate that their homophilic engagement during cognate TCR-CD1d interaction is critical to NKT cell development.

DISCUSSION

TCR and CD4-CD8 coreceptor signaling events are central to the selection and lineage differentiation of thymocytes. However, the characterization of new lineages recognizing ligands presented by cells as divergent from thymic epithelial cells as cortical thymocytes has suggested that unidentified, cell-type specific signals might also decisively impact the selection and differentiation events. The present study identified two members of the SLAM family of receptors, Slamf1 and Slamf6, that are highly expressed by cortical thymocytes and control the transition between positive selection and the subsequent expansion and differentiation of the NKT cell lineage. NKT cell precursors lacking either Slamf1 or Slamf6 exhibited only modest defects. However, in mixed bone-marrow chimeras designed to abrogate both Slamf1 and Slamf6 signaling during CD1d ligand recognition in the thymus, a stronger developmental arrest was observed, demonstrating that Slamf1 and Slamf6 exerted essential and intrinsic but partly redundant effects through homophilic self-association across the cell-cell synapse. The demonstration that Slamf1 and Slamf6 could exert partially redundant effects is consistent with their shared recruitment of SAP and Fyn and might suggest an explanation for the past failure of investigators to ascribe the multiple phenotypes associated with the SAP or Fyn deficiencies to individual members of the SLAM family of receptors (Veillette, 2006). Deficiencies of Slamf1, Slamf6, or their intracellular signaling partners Fyn and SAP affected NKT cell development with increasing severity. It is possible therefore that the residual NKT cells developing in mixed Slamf1 + Slamf6 "pseudo-double KO" chimeras have signaled through other, less prominent SLAM family receptors, such as Slamf3 (Ly9, CD229), Slamf5 (CD84), or Slamf7 (CRACC, CD319), for example. Alternatively, bystander engagement of Slamf1 and Slamf6 by third-party cortical thymocytes (not directly involved in TCR-CD1d ligand interactions) might have provided the required signals, albeit inefficiently. This issue will be resolved when double and triple mutants of the SLAM family of receptors become available.

All mutations impacted the same stage of NKT cell development, further supporting a linear model of signaling through SLAM family receptors and intracellular SAP and Fyn. Thus, whereas CD24^{hi}CD69^{hi}Tetr^{+} cells, representing the stage just after positive selection, were clearly identified, the subsequent intrathymic expansion and differentiation of NKT cells was invariably defective. These results stand in contrast with a previous conclusion that Fyn acted upstream of TCR expression on the basis of the apparent restoration of NKT cells in Fyn-deficient mice upon the expression of a Vα14-Jα18 transgene (Gadue et al., 2004). The reasons for this discrepancy are unclear, but our own experiments clearly demonstrated that NKT cell development was severely quantitatively impaired in Vα14-Jα18 transgenic mice lacking either Fyn or SAP. One possibility is that the overexpression of the TCR transgene combined with the leakiness of the Fyn mutation might have resulted in the apparent "rescue" of NKT cells in the discordant report. Another, nonexclusive possibility is that the rescued cells in these transgenic mice corresponded to a subset of tetramer-positive, CD1d-independent, non-NKT lineage cells, which used a prematurely expressed Vα11 promoter to drive Vα14-Jα18 expression (Bendelac et al., 1996; Wei et al., 2006). These cells are not present in our new transgenic lines that use a CD4 promoter. In any case, the present combined results render the previous conclusion that Fyn was involved prior to TCR engagement speculative and, instead, support a unified model in which the bifurcation of the NKT cell lineage is driven by the peculiar signals emanating through SLAM family receptors, SAP and Fyn at the intercellular synapse formed during the thymic selection events. These findings help focus the search for the mechanisms of developmental arrest during the events associated with CD1d ligand recognition rather than during some defective TCR arrangement or lineage precommitment. The arrested cells did not exhibit signs of increased apoptosis and could not be rescued by a Bcl-xL transgene driven by a proximal Lck promoter (data not shown), suggesting that, rather than a mere lack of a survival signal, a defect in positive selection, a block in proliferation, or an increased negative selection might also underlie the developmental defect.

Signaling through SAP and Fyn is reported to activate SHIP1, Dok1 and Dok2, and RasGap and to inhibit Ras signaling (Veillette, 2006). Fyn also activates the NF-kB

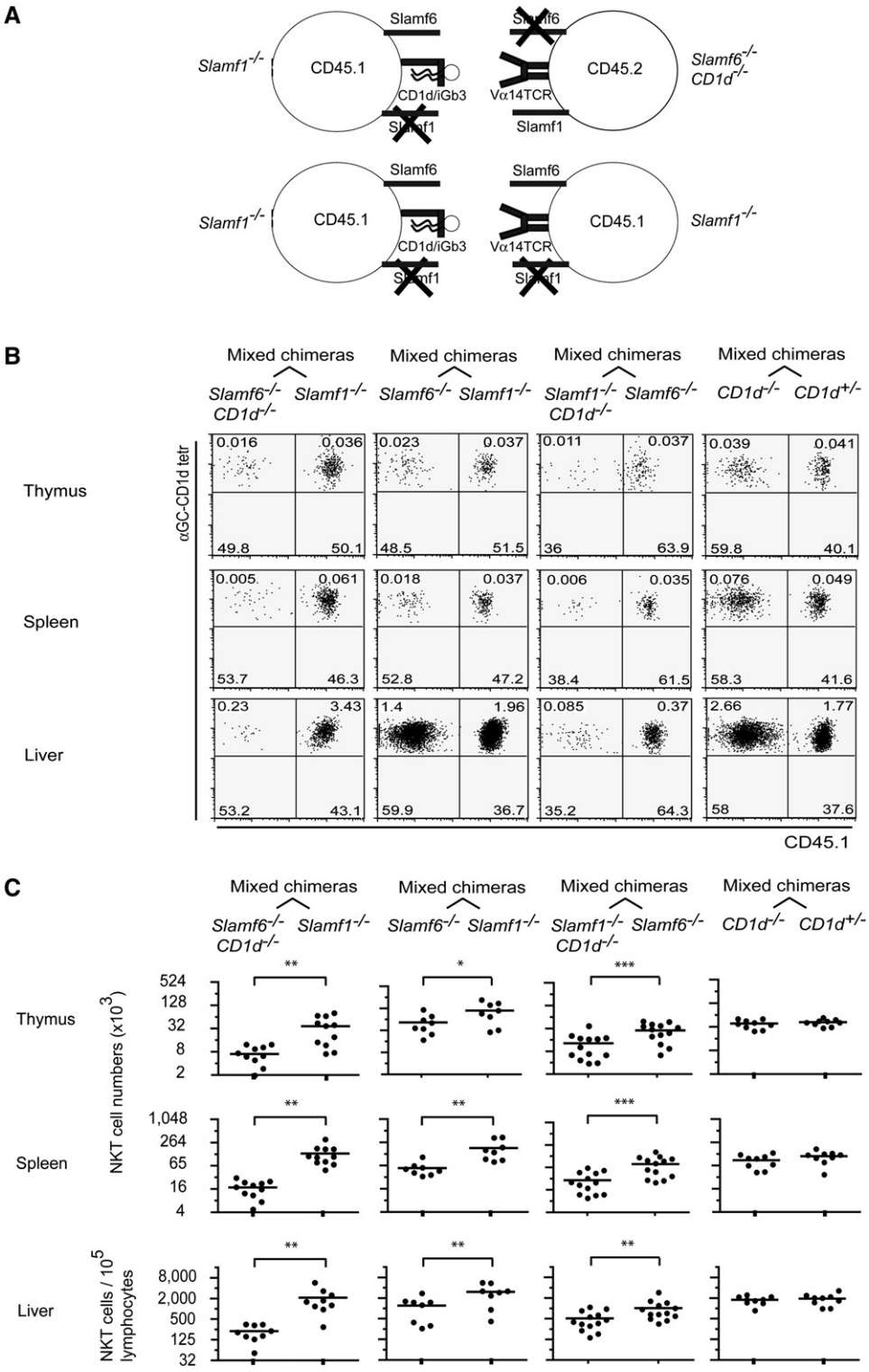

Figure 5. NKT Cell Developmental Block in Pseudo-Double KO Chimeras

(A) Cell interaction schemes illustrating the functional double deficiency created in the *CD1d*$^{-/-}$ compartment of mixed chimeras of the *Slamf1*$^{-/-}$ + *Slamf6*$^{-/-}$*CD1d*$^{-/-}$ (top) versus the single deficiency in the *CD1d*$^{+/+}$ compartment (bottom). The *Slamf6*$^{-/-}$*CD1d*$^{-/-}$ NKT cell precursors must interact with *Slamf1*$^{-/-}$ thymocytes, the sole source of CD1d ligands, functionally removing both Slamf1 and Slamf6 signals during TCR engagement (top), whereas the *Slamf1*$^{-/-}$ NKT cell precursors must interact with *Slamf1*$^{-/-}$ thymocytes, creating a single KO situation (bottom).

(B) Mixed radiation bone-marrow chimeras as indicated. FACS dot plots are gated on CD1d-αGC+ NKT lineage cells and show the expression of the CD45.1 allelic marker in tetramer-positive and -negative cells in different tissues, as indicated.

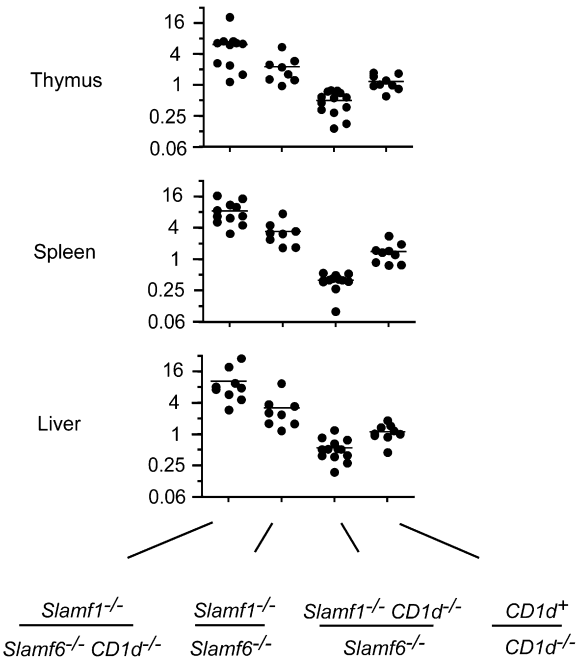

Figure 6. Relative Contribution of Slamf1 and Slamf6 in Pseudo-Double KO Chimeras

Summary scatter plot showing the calculated ratios of NKT cells found in the two bone-marrow-derived compartments of individual chimeras in Figures 5B and 5C. A two-way ANOVA test showed statistically significant differences between the ratios found in different chimera sets.

pathway (Cannons et al., 2004), whose importance for NKT cell development, particularly for survival, is well established (Schmidt-Supprian et al., 2004; Sivakumar et al., 2003; Stanic et al., 2004). The coordinated expression of CD1d and SLAM-family members in mouse and human thymocytes, segregated from MHC class I and class II in thymic stromal cells, favors the spatial and temporal co-signaling of SLAM family members along with the TCR during NKT cell development. Notably, because Slamf1 and Slamf6 signaling appears to be required concomitantly with TCR engagement, it would not be available to mainstream thymocytes because they interact with MHC ligands on stromal cells (which do not express these SLAM family members). Thus, our findings suggest that the peculiar thymic expression patterns of MHC, CD1d, and SLAM family glycoproteins in mouse and human dictate alternative lineage fates. The topography of ligand expression defines therefore a specialized niche that provides the necessary signals for divergent lineage differentiation.

Interestingly, recent reports have suggested that the inactivation of the Tec kinases, which signal downstream of the TCR, induced a shift from the conventional T cell to a memory or effector NK1.1^{+} "innate-like" T cell lineage through the induction of eomesodermin and the interleukin-2 (IL-2) receptor β chain (Atherly et al., 2006; Broussard et al., 2006), a striking parallel to the induction of T-bet, a homolog of eomosodermin, and the IL-2 receptor β chain required for the NKT cell lineage (Intlekofer et al., 2005; Matsuda et al., 2006; Townsend et al., 2004). The development of these MHC-restricted innate-like T cells required MHC class I expression on bone-marrow-derived rather than epithelial cells. Likewise, normal or transgenic expression by thymocytes of MHC class Ib or MHC class II, respectively, resulted in the development of memory or effector type T cells (Choi et al., 2005; Li et al., 2005; Urdahl et al., 2002). These intriguing findings might indicate that SLAM family receptor signaling is generally involved in the formation of memory or effector innate-like lineages by bone-marrow-derived thymocytes and suggest novel mechanisms of lymphocyte lineage instruction based not only on TCR and coreceptor signals but also on accessory signals specifically provided by different antigen-presenting cell types. Indeed, recent findings indicate that SAP is essential for the development of these innate-like lineages, as well (Horai et al., 2007; Li et al., 2007 [both in this issue of *Immunity*]).

Notably, polymorphism at the SLAM locus, in particular the differential expression of Slamf6 splice variants with differential signaling properties, is associated with autoimmunity and lupus (Kumar et al., 2006; Wandstrat et al., 2004). The absence of Slamf1 expression on thymic DP cells was recently reported in nonobese diabetic (NOD) mice, a phenotype correlating with homozygosity for the *SLAM*^{NOD} locus in genetic analysis (Jordan et al., 2007). The link between Slamf1 and variations in NKT cell numbers remains correlative, but it is consistent with a previous mapping of NKT cell deficiency in this autoimmune strain identifying two independent loci, *Nkt1* and *Nkt2* (Jordan et al., 2007; Rocha-Campos et al., 2006), because the SLAM locus is a candidate for *Nkt1*. Although *Nkt1* congenic NOD mice did not show the amelioration of diabetes (Rocha-Campos et al., 2006), the NKT cell increase in these mice was modest, and the role of NKT cells in type I diabetes remains intriguing (Delovitch and Singh, 1997).

Although previous research emphasized the role of SLAM family receptors in multiple forms of innate and adaptive immune responses, our findings reveal a new essential role of the SLAM family of receptors in lymphocyte development and further suggest a more general mechanism of T cell lineage differentiation through the topologically segregated expression of TCR ligands and costimulatory receptors. The results also strengthen the emerging concept that polymorphism of the SLAM locus might underlie important genetic variations in the development or function of entire populations of immune cells to fine-tune the balance between the effector and regulatory pathways that control infection and autoimmunity.

(C) Summary scatter plots show NKT cell numbers in different tissues and in CD45 allele-marked compartments of individual mixed chimeras. "*," "**," and "***" indicate statistical significance ($p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively) with a t test for paired comparisons of the two hemopoietic compartments in individual mixed chimeras. Results are pooled from two to three sets of chimeras showing similar results (ANOVA).

EXPERIMENTAL PROCEDURES

Mice

Slamf1$^{-/-}$ (Wang et al., 2004) mice were used after eight backcrosses to C57BL/6, *Slamf6*$^{-/-}$ (Howie et al., 2005) (lacking exons 2–3 encoding the entire extracellular domain) after six backcrosses, *Slamf2 (Cd48)*$^{-/-}$ after 12 backcrosses, *Sh2d1a (SAP)*$^{-/-}$ (Wu et al., 2001) and *Fyn*$^{-/-}$ (Stein et al., 1992) after ten backcrosses, and *Jα18*$^{-/-}$ (Cui et al., 1997) and *CD1d*$^{-/-}$ (Carnaud et al., 1999) after > 12 backcrosses. *Slamf4 (2B4)*$^{-/-}$ were on a pure C57BL/6 background (Vaidya et al., 2005). C57BL/6 (CD45.2) and B6.SJL-*Ptprca Pep3b*/BoyJ (CD45.1) were from Jackson Labs. We verified that *Slamf1*$^{-/-}$, *Slamf6*$^{-/-}$, and *Slamf2*$^{-/-}$ mice shared the same SLAM locus of 129 origin by using strain-specific antibodies against Slamf3 (Ly9) and Slamf4 (CD244, 2B4) and by genotyping for a SNP rs315321197 located at the other end of the locus (data not shown). For the generation of the Vα14-Jα18 transgenic mice, the prerearranged Vα14-Jα18 TCR α chain complementary DNA (cDNA) of the DN32.D3 hybridoma (Bendelac et al., 1996) was inserted into the SalI site of a plasmid containing the minimal CD4 promoter and enhancer and the intronic silencer (Sawada et al., 1994). The linearized (via NotI) construct was injected into fertilized C57BL/6 oocytes, and the injected oocytes were implanted into pseudopregnant CD-1 (VAF+) outbred female mice. Transgenic mice were screened with polymerase chain reaction (PCR) (forward primer: 5′-TGTAGGCTCAGATTCCCAACC-3′; reverse primer: 5′-GAGGATGGAGCTTGGGAGTCAGG-3′) and were bred onto various gene-deficient backgrounds. All mouse work was done in accordance with the guidelines of the Institutional Animal Care and Use Committee at the University of Chicago.

Antibodies and Flow Cytometry

Anti-Slamf6 (clone 13G3) was generated from the spleen of a *Slamf6*$^{-/-}$ mouse immunized with wild-type thymocytes (S.R., unpublished data) and anti-Slamf5 mAb (a generous gift from Pablo Engel) was reported (Romero et al., 2005). CD1d-αGalCer tetramers were prepared as previously described (Benlagha et al., 2000). Fluorochrome labeled monoclonal antibodies (clone indicated in parentheses) against Slamf1 (9D1), Slamf3/CD229.1/Ly9.1 (30C7), Ep-CAM (G8.8), Ly51 (6C3), NK1.1 (PK136), CD44 (IM7), CD24 (M1/69), B220 (RA3-6B2), CD8α (53−6.7), CD4 (RM4-5), CD45 (30-F11), CD11c (HL3), CD11b (M1/70), CD1d (1B1), CD45.1 (A20), rat κ light chain (MRK1), and were purchased from eBioscience or BD Biosciences. The FITC-conjugated anti-Syrian and Armenian hamster IgG cocktail (G70-204, G94-56) was purchased from BD Biosciences. For NKT cell enrichment, thymocytes were stained with APC-conjugated CD1d-αGalCer tetramers, bound to anti-APC paramagnetic beads and positively selected with an autoMACS (Miltenyi biotech) as described (Benlagha et al., 2005). All steps were performed at ice-cold temperature. Samples were analyzed on BD Canto or LSRII flow cytometers. Dead cells were excluded with DAPI staining and doublets by gating on FSC and SSC area, height and width.

Generation of Mixed Bone-Marrow Chimeras

Four to eight-week old *Jα18*$^{-/-}$ mice were subjected to 900 Rads irradiation using a gamma cell 40 irradiator with a cesium source. Four to eight hours later, irradiated mice were injected intravenously with $5x10^6$-10^7 bone marrow cells isolated from femurs of donor mice, and depleted of T cells by magnetic cell sorting (autoMACS, Miltenyi biotech) using CD3-PE antibody (eBiosciences) and anti-PE beads. Mice were analyzed six to eight weeks after reconstitution.

Quantitative Real-Time PCR of Canonical Vα14-Jα18

$CD4^+CD8^+CD24^{hi}$ thymocytes were sorted from C57Bl/6, *CD1d*$^{-/-}$, *Sh2d1a*$^{-/-}$, *Fyn*$^{-/-}$, and *Jα18*$^{-/-}$ mice on FACSAria (BD Biosciences) and MoFlo (Dako Cytomation) cell sorters. Total RNA of 5×10^5 sorted thymocytes was extracted into 10 μl and treated with DNase1 with the RNAqueous Micro Kit (Ambion). Eight microliters of each DNase1-treated RNA sample was primed with oligo-dT and reverse transcribed into a 20 μl volume with the SuperScript III Platinum Two-Step qRT-PCR kit (Invitrogen). Vα14 and Cα-specific primers, as well as the Cα-specific Taqman probe, were previously described. A probe specific for the sequence spanning the NKT cell clonotypic Vα14-Jα18 junction (5′-FAM-CTGTGTGGTGGGCGATA-MGBNFQ-3′) was purchased from Applied Biosystems. PCR reactions were run in an ABI Prism 7700 Sequence Detector (Applied Biosystems). Each duplicate reaction contained 5 μl cDNA, 0.2 μM each primer and Taqman probe, and Platinum Quantitative PCR Supermix-UDG with ROX reference dye (Invitrogen) to a final volume of 50 μl. Initial 2 min holds at 50°C and 95°C were followed by 50 cycles consisting of 15 s at 95°C and 45 s at 60°C. Amplification was analyzed with ABI Prism Sequence Detection Software Version 1.9.1 (Applied Biosystems) and the Standard Curve Method described in the ABI Prism 7700 Sequence Detection System User Bulletin #2. Ten-fold serial dilutions of a plasmid containing a rearranged Vα14 TCRα cDNA were used for Vα14 and Cα standard curve generation.

Immunohistochemistry

Thymi from 4-week-old C57Bl/6 mice were rinsed with phosphate-buffered saline (PBS) and frozen in optimal cutting temperature (OCT) freezing medium (Tissue-Tek, Sakura Finetek) by flotation on 2-methylbutane (Sigma-Aldrich) over dry ice. Frozen thymi were stored at −80°C. Thymic sections were cut at 6 mm on a LEICA microtome, fixed with aceton for 5 min, and stained with purified anti-Slamf1 (9D1) and a Cy3 TSA amplification system (Perkin Elmer) or biotinylated anti-Slamf6 (13G3) and then streptavidin-rhodamin- (Molecular Probes) and fluorecein isothiocyanate (FITC)-conjugated anti-Ly51 (6C3) (a marker for cortical epithelium). Images were captured on a Leica SP2 A OBS Laser Scanning Confocal and a Zeiss Axiovert 200 immunofluorescence microscope and processed with LCS Leica Confocal Software, Openlab software, and Adobe Photoshop.

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